

## In Vitro Evaluation of Binding of Fish Mucus by Nanoparticles Induced Oxidative Stress on the Common Carp

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**Abstract:** Common carp (*Cyprinus carpio*) is a large, deep bodied fish and can tolerate all types of water. Fish skin mucus comprises numerous immune substances that provide defense against a wide spectrum of pathogens. The present research project is designed to study the binding of fish mucus isolated from common carp with silver nanoparticles. The characterization was done through FTIR and UV visible spectrophotometer. The peak of mucus based silver nanoparticles was higher than that of crude mucus extract in UV-visible spectrum. The FTIR spectra showed different functional groups such as alkanes, alkyl halides and amines. The different biological activities like antimicrobial, antioxidant and antibiofilm potential of fish mucus based nanoparticles were checked. For some activities, mucus exhibited better results and for others mucus based silver nanoparticles had higher activities. Fish mucus of common carp (*Cyprinus carpio*) showed highest antibacterial activity against *E. coli* bacteria with inhibition zone of 21 mm in crude mucus case. Lowest antibacterial activity was found against the *B. subtilis* with inhibition zone of 9 mm. Biochemical analysis exhibited higher values of catalase activity (CAT), superoxide dismutase activity (SOD), peroxidase activity (POD) and total soluble protein (TSP) for mucus and lowest for free silver nanoparticles.

**Keywords:** *Cyprinus carpio*, POD, SOD, TSP.

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### Introduction

Common carp is a large, deep bodied fish. Carp color is varied silver to olive green, on the sides and back brass and grey. Lower fins are orange red, belly is yellowish. Its cheeks and gill cover are

partially scaled and a single dorsal spine. Near the corners of its mouth whisker like appendages called barbells are present. All types of bases and water clarity fluctuating from clear to murky can tolerate. Carp are one of the most damaging aquatic invasive species due to its wide distribution and severe impacts in shallow lakes and wetlands (Joseph R. Tomelleri., 2018).

Slimy slippery layer covered the epithelial surface called mucus. It contain enzyme, protein or water. The skin is continually noticeable to pathogens attacks, because water is a perfect medium for bacteria and parasitic microbes (Wang *et al.*, 2011). Mucus serve as a mechanical and biochemical barrier. Carbohydrate binding proteins and lactins are present in mucus and might function in pathogens defense that are neither enzyme nor antibody. In the fish skin mucus several humeral defense factors are present as immunoglobulin, C reactive protein, compliment, lysozyme, and hemolysin (Tateno *et al.*, 2011). Particles size range between 1 to 100 nm called nanoparticles within interfacial layer. This layer is an integral part and effects all its properties. Organic, inorganic molecules or ions present in interfacial layer. Inorganic molecules are coated by organic molecules are known as stabilizer, passive agents, surface and passive ligands. Optical properties are often possess by nanoparticles as they are small enough to restrain their electrons and express quantum effects. They are able to move across the cell membrane in organism. Nanoparticles are a link between bulk materials and atomic structure. They have high surface area and volume ratio provide a driving force for diffusion. (Batista *et al.*, 2015).

To the best of the authors' knowledge, there is little work on the binding of mucus with nanoparticles. Therefore the present work was undertaken with the main objective to investigate the binding of common carp (*Cyprinus carpio*) mucus with silver nanoparticles. Different biological activities like antimicrobial, antioxidant, antibiofilm and cytotoxicity of the mucus and nanoparticles was also investigate.

## Material method

### Sample Collection:

Fish mucus samples of *Cyprinus carpio* were collected from the fish farm of Zoology Department, University of Agriculture Faisalabad. Different sizes of carp fish were captured from the pond for the purpose of mucus collection.

### Collection of mucus:

The mucus of fish was collected, with the help of spatula directly from the upper side of the fish, not collected from the lower side in order to prevent from the contagion of urine and sperms. For identification, according to the weight of the sampling, the mucus samples were located in eppendorf tubes. These mucus samples were instantly by kept it into box of crushed ice at -20°C and shifted to Medicinal Biochemistry Laboratory, Department of Biochemistry, from Department of Zoology Wildlife and Fisheries, University of Agriculture Faisalabad for more studies.

### Preparation of crude mucus sample:

The extract of crude mucus was prepared from the earlier conserved mucus of fish. Skin mucus saved from the fishes for crude mucus extract and then centrifuge at 1500 rpm for 15 minutes. The supernatant was collected to quantitative qualitative assays for the evaluation of the biochemical components. (Tyor and kumara, 2016).

### Preparation of silver oxide solution:

For the silver oxide preparation, AgO salt of 0.069g was measured by analytical balance and mixed with distill water and make total volume 100m.

**Synthesis of Mucus based Ag Nanoparticles:**

Silver oxide solution and the purified mucus of common carp (*cyprinus carpio*) were used for the preparation of mucus based Ag nanoparticles. All the solutions were prepared in distilled water. Then, 0.5% (w/v) of homogenous mucus solution was prepared and the concentration of silver nitrate was 1mm. The mucus based Ag nanoparticles were synthesized by autoclaving the solution at 121<sup>0</sup>C for 15 min (Munir *et al.*, 2016)

The further details of this process are as below;

**(1) Biological activities of fish mucus, Ag Nanoparticles and Mucus Based Ag Nanoparticles:**

From the fish skin mucus extract, antibacterial evaluation were determined by agar well diffusion

**Antibacterial activity:**

Antibacterial activity was measured against gram positive (*Bacillus subtilus*) and gram negative (*Escherichia coli*) through ager well diffusion method (Cavalieri *et al.*, 2005).

**Antifungal Activities:**

Antifungal activity of the skin mucus of fish sample was performed against the two fungal strains by ager well diffusion method (Phillott and Parmenter, 2012).

**Minimum Inhibitory Concentration (MIC):**

In microbiology, minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial (like an antifungal, antibiotic or bacteriostatic) drug that will inhibit the visible growth of a microorganism after overnight incubation (Wiegand *et al.*, 2008).

**(2) Antioxident activity:**

Antioxident activity of fish mucus was determined by using following various antioxidant methods.

**(a) Reducing power:**

According to the method of Garcia *et al*, (2014), the redusing power of skin mucus sample of fish was measured.

**(b) Total phenolic content (TPC) Quantification of fish mucus:**

Total phenolic contents (TPC) of fish mucus, mucus bound nanoparticles and Ag nanoparticles were measured through the process of Mamelona *et al.* (2007).

In the gallic acid equivalents (GAE) total contant of phenolic (TPC) compounds were determined through the following formula.

$$T-C \times V / M$$

Where T – total contents of phenolic compound in mg GAE of mucus extract, C- the concentration of gallic acid calculated from calibration curve in mg/mL, V- the volume of mucus extract in mL and M- the weight of fish mucus extract in grams.

**(c) Total flavonoid content (TFC) Quantification of fish mucus:**

The total flavonoid contents (TFC) of epidermal mucus, Ag nanoparticles and mucus bound nanoparticles was measured through the method of (Chang *et al.*, 2006).

**(d) 2,2-Diphenyl-1-Pierylhydrazyl (DPPH)**

For the determination of DPPH, method determined by Garcia *et al.* (2014) was pursued. As a standard Ascorbic acid was used. The DPPH activity was measured by following formula;

DPPH inhibition activity (%) =

$\text{Ablank} - \text{Asample} / \text{Ablank} \times 100$

Where Ablank = Absorbance of blank and Asample = Absorbance of sample

### (3) Inhibition of microbial biofilm:

Microbial biofilm inhibition, against *Escherichia coli* and *Bacillus subtilis* was performed according to the process of Shahid *et al.* (2015) with the little modification. The inhibition of microbial biofilm was measured by the following formula:

$$\text{inhibition \%} = 100 - \frac{\text{Optical density of sample}}{\text{Optical density of negative control}} \times 100$$

### (4) Cytotoxic activity:

Cytotoxicity of fish mucus extracts were determined by hemolytic assays.

#### Hemolytic activity:

In EDTA, blood samples were collected which was diluted with saline (0.8% NaCl) and centrifuged at 15000 for 15 minutes. The RBCs or erythrocytes isolated and then dilute in phosphate buffer saline (PBS) having pH of 7.4 and made the suspension. Then added mucus extract in it and incubated for 10 minutes at room temperature which were inducing membrane lipids oxidative degradation. PBS was the negative control and Triton-X was taken as the positive control. The absorbance was measured at 540nm by spectrophotometer Powell *et al.* (2000).

Following formula are used for measuring the hemolytic activity:

% age of hemolytic activity-  $\frac{\text{Absorbance of sample} - \text{Absorbance of negative control}}{\text{Absorbance of positive control}} \times 100$

### (5) Biochemical analysis:

Catalase (CAT), superoxide dismutase (SOD) and peroxidase (POD) assays and protein estimation were used for biochemical analysis of fish mucus.

#### (a) Catalase activity (CAT)

The proposed method Mohebbi *et al.* (2012) was used to measure the activity of catalase. The hydrogen peroxide was used as a substratum in this method and catalase enzymes were used to detect hydrogen peroxide decomposition using UV spectrophotometer.

#### (b) Superoxide Dismutase (SOD)

SOD activity was evaluated with slight changes in accordance with the Kumari *et al.* (2014) process. SOD activity was analyzed in terms of enzyme comparative activity as protein U / mg.

#### (c) Peroxidase activity (POD)

The activity of peroxidase (POD) as a hydrogen donor was measured by using guaiacol. Change was determined at 470 nm of absorbance for 1 minutes. The activity of enzyme was assayed as unit (absorbance at 470 nm defines one activity unit, changes of 0.01 per minutes) and per gram of fresh weight of the tissues. (Jayaseelan *et al.*, 2014).

**(d) Protein estimation**

Hiwarale *et al.* (2016) method was used to measure the protein of fish epidermal mucus samples as a standard Bovine serum albumin (BSA) was used. The sample absorbances were measured at 600 nm through the spectrophotometer.

**(6) Characterization:****(a) UV visible spectroscopy:**

Field UV absorbance measurements were done through the process of as same to that determined by Zamzow *et al.* (2004). From each fish species the outer mucus of body surface was sampled as the day of capturing and optic fiber spectrometer was used to measure the UV absorbance of fish mucus and silver nanoparticles. The fish mucus was softly removed off from the whole dorsal side of fish through the deadly blade, as mentioned by Zamzow *et al.* (2006). To analyze the field absorbance of UV, mucus was kept on the ultraviolet clear microscopic slide. The mucus sample thickness through the help of cover slips (0.26 mm thickness) was standardized by its adhesion to two sides of slides that worked as spacers. An optic fiber cable (Optics Oceans, diameter of 1000 nm) attached to the source of UV light that was placed into the metal holder. It was generally having the lens therefore used to light up to the sample when it was set into the slot of microscope.

**(b) Fourier transformed infrared spectroscopy (FTIR):**

For spectroscopic study of solid part of the fish mucus, Fourier transformed infrared spectroscopy (FTIR) is used. 110 mg of desiccated potassium bromide (KBr) was added with the sample and for further reading the spectrum, condensed to prepare as a salt disc like. In to the general transmission cell, the mucus was very thick for injection. The occurrence of calcium fluoride, water vapours and liquid water bands that disturbs the spectral profile. The fish mucus, mucus based nanoparticles analysis of FTIR exhibited diverse spectral bands and other functional groups such as the occurrence of the aromatic compounds, aliphatic alkyl group, and carbohydrates, primary amines and halides (Bragadeeswaran *et al.*, 2011).

**(7) Statistical analysis:**

For statistical analysis, simple mean and standard deviation was applied. Usually, bar graphs were used to express the data to examine the study hypothesis for the characteristics of interest.

**Results****(1) Antioxidant Activity of Crude Fish Mucus and Mucus Bound with Ag Nanoparticles:**

Antioxidant activity of fish mucus was determined through various assays.

**(a) Total Phenolic Contents (TPC):**

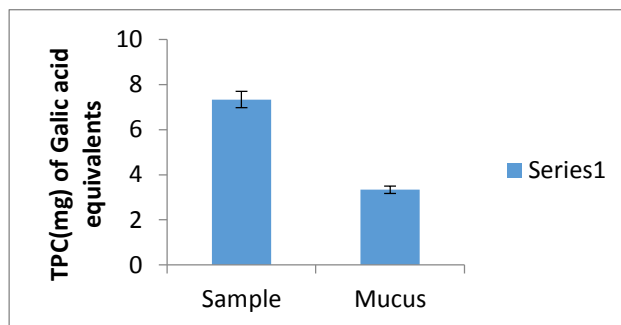
Total phenolic contents of plant extract are higher than that of fish mucus and mucus based Ag nanoparticles. Fish mucus has lower phenolic contents and its antioxidant activity is also low. Total phenolic contents of mucus extract were evaluated by using Folin-Ciocalteu colorimetric procedure, and regression equation of gallic acid calibration curve was used for this purpose. The amount of phenolic per each extract was expressed as gallic acid equivalent. The results obtained from the assay were expressed as mean  $\pm$  standard deviation of triplicate analyses and are presented (Turkoglu *et al.*, 2010a).



**Table 4.1: Total phenolic contents of mucus and mucus based Ag nanoparticles. Given below is the data of triplicates  $\pm$  SD.**

Sample	TPC
Mucus	7.33 $\pm$ 0.22
Mucus based Ag nanoparticles	3.33 $\pm$ 0.51

In my research work, total phenolic contents were also measured by Folin-Ciocalten method as this method is fast and simple way for quick determination of sample's phenolic contents. Many earlier reports were found related to the use of this Folin-Ciocalten reagent (Yadav *et al.*, 2014; Souza *et al.*, 2008; Kumar and Chattopadhyay, 2007).



**Fig 4.1** Comparison of total phenolic contents crude mucus and mucus based Ag nanoparticles. Given data is the average of three replicates  $\pm$  S.E.

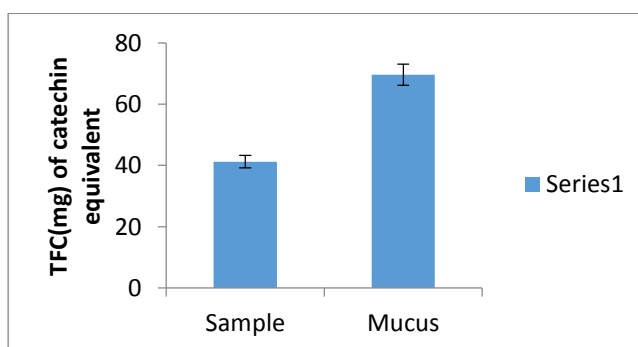
The above graph shown that the total phenolic contents of mucus extract of carp is less than that of mucus based Ag nanoparticles.

#### (b) Total Flavonoids Content (TFC):

In natural compounds, the flavonoids are the vital group, containing vegetables, fruits and cereals. Due to their wide spectrum of biological and chemical activities, including free radical scavenging properties, flavonoids are the most likely important phenolics. Flavonoids are also therapeutic agents against large number of diseases (Glucin, 2005; Turkoglu *et al.*, 2010a). TFC of mucus extract and mucus based Ag nanoparticles were calculated as catechins equivalents.

**Table 4.2: total flavonoid contents of mucus and mucus based Ag nanoparticles**

Sample	TFC
Mucus	69.60 $\pm$ 1.28
Mucus based Ag nanoparticles	41.23 $\pm$ 1.18



**Fig 4.2** Compration of total flavonoid content in crude mucus and mucus based Ag nanoparticles

Figure 4.3 Explains how quality of flavonoids varies in different extracts of fish mucus. High flavonoid contents were found in crude mucus extract of fish as compared to the Ag nanoparticles.

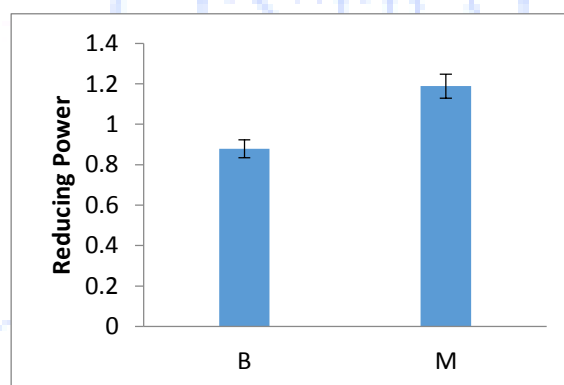
### (c) Reducing Power:

The reducing power assay is mostly used to determine the capability of an antioxidant to give an electron due to the reducing capacity of a compound. It is the major indicator of antioxidant activity (Duan *et al.*, 2007).

For the determination of the sample extract, ability to reduce iron (III) reducing power assay is used. This reducing power assay depends upon the concentration for all samples. Increase in the reducing power of sample or mixture means that there is an increase in absorbance of reaction mixture. The sample which has high reducing power means higher ability to donate electrons and  $\text{Fe}^{3+}$ / ferreic cyanide complex reduced into the ferrous form formation of blue color. The color of test sample changes from yellow to green or blue depends on reducing power of sample. Absorbance was measured at 700nm.

**Table 4.3: Reducing power of mucus and mucus based Ag nanoparticles**

Sample	Reducing power
Mucus bound Ag nanoparticles	0.87
Mucus	1.18



**Fig. 4.3:** Graphical representation of reducing power of mucus and mucus based Ag nanoparticles

The results observed that the reducing power of mucus bound with Ag nanoparticles was less than the reducing power of crude mucus because the mucus having bound Ag nanoparticles was concentrated than that of crude mucus so the absorbance of mucus based Ag nanoparticles is high.

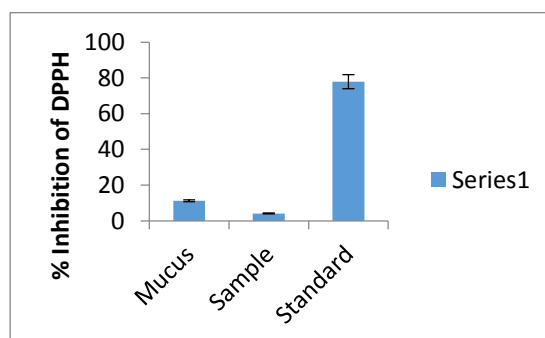
### (d) Free Radical Scavenging Activity (DPPH):

DPPH is a well-known free radical which gives strong absorption band at 517nm. The color of DPPH solution is deep violet and its color disappears and changes to yellow when neutralized by antioxidant compound. Free radical scavenging activity of mucus extract was determined by DPPH scavenging assay.

The scavenging activities of all samples were concentration dependent. Lower absorbance of the reaction mixture indicated higher DPPH radical scavenging activity (Gulcin *et al.*, 2006)

**Table 4.4: the free radical scavenging activity of mucus and mucus based Ag nanoparticles. The data below is the value of triplicates $\pm$ SD.**

Sample	DPPH Activity
Mucus	11.14 $\pm$ 0.07
Mucus based Ag nanoparticles	0.12 $\pm$ 0.04
Standard	77.89



**Fig. 4.5:** Graphical representation of free radical scavenging activity (DPPH)

The DPPH scavenging activity of different mucus extracts have been shown in figure. DPPH activity of crude mucus is higher than of mucus based Ag Nanoparticles.

## (2) Biofilm Inhibition:

Biofilm is a thin layer of mucilage adhering to a solid surface. It comprises group of microorganisms in which cells are attached to the surface. These cell becomes surrounded within a slimy extracellular matrix that is composed of extracellular polymeric substances such as DNA, proteins and polysaccharides (Sutherland, 2005; Flemming and Wingender, 2010).

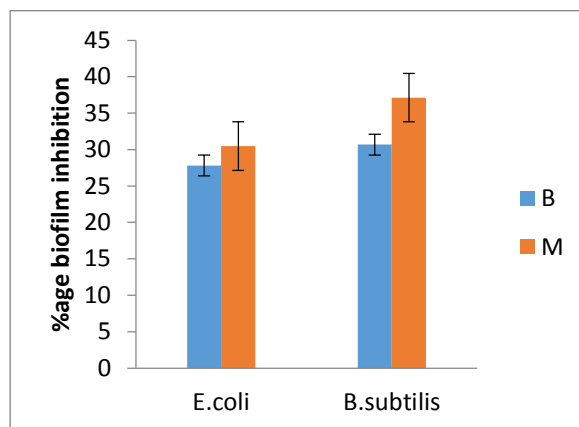
In the biofilm form, bacteria are more resistant to various antimicrobial treatments. Bacteria in a biofilm can also survive harsh conditions and withstand the host's immune system. The purpose of this activity was to find out the potential of fish mucus extract and mucus based Ag nanoparticles to inhibit biofilm formation. At first, both samples were tested for their antibiofilm activity and results are given in table.

The resistance of biofilm is due to the occurrence of some polysaccharides and enzymes that cause the molecules inhibition or receptor inhibition in the pathway of quorum (necessary for formation of biofilm). Lectins are important for colonization and bacterial infection and also play significant role in formation of biofilm which have been inhibited by the polysaccharides (Valle *et al.*, 2006; Rendueles *et al.*, 2013).

**Table 4.5 Inhibition of *Bacillus subtilis* and *E.coli* biofilm by the mucus extracts and mucus based nanoparticles**

Sample	<i>E.coli</i> mean $\pm$ SD	<i>B.subtilis</i> mean $\pm$ SD
Mucus	30.47 $\pm$ 1.029	37.13 $\pm$ 0.54
Mucus based sample	27.82 $\pm$ 1.04	30.69 $\pm$ 0.83





**Fig 4.6** Inhibition of *Bacillus subtilis* and *E.coli* biofilm by the mucus extracts of Common carp fish and mucus based Ag nanoparticles. Given data is average of three replicates  $\pm$  S.D.

In silver oxide nanoparticles, the biofilm inhibition against *E. coli* is more than that of inhibition against *Bacillus subtilis*. Whether in case of pure mucus extract the biofilm inhibition against *Bacillus subtilis* is more than against the *E. coli*.

### (3) Antibacterial activity:

Fish mucus extracts were tested for their antimicrobial activities. For this activity two bacterial culture were selected. *E. coli*, *B. subtilis*, these two bacterial cultures or stains were used in antibacterial activity.



**Fig 4.7(a)** Zone of inhibition of crude mucus and mucus based Ag nanoparticles against *B. subtilis*. Zone of inhibition of positive and negative control. **(b)** Zone of inhibition of crude mucus and mucus based Ag nanoparticles against *E. coli*. Zone of inhibition of positive and negative control.

Above figures demonstrated that fish mucus of common carp (*Cyprinus carpio*) showed highest antibacterial activity against *E. coli* bacteria with inhibition zone of 15 mm in crude mucus case. Lowest antibacterial activity was found against the *B.subtilis* with inhibition zone of 14 mm. The crude mucus show more antibacterial activities in all bacterial cultures (in all cases). Ampiciline was used as a positive control to compare the bacterial zones of fish mucus.

**Table 4.6 Antibacterial activity against different bacterial of fish (Common carp) mucus, mucus based nanoparticles and ampiciline**

Sample	<i>E. coli</i> (mm)	<i>B. subtilis</i> (mm)
Mucus	21	18
Sample	10	9
Ampiciline	29	24

The native fish species like common carp and *Catla catla* showed highest antimicrobial activity rather than that of foreign fish species like *Ctenopharygodon idella* and *Hypophthalmichthys molitrix* (Balasubramanian *et al.*, 2012).

Antibacterial proteins are secreted by the fish that made the fish able to permeabilize the target cell membrane and in this way perform as a protection obstacle. Antibacterial activity is due to the antibacterial glycoproteins that are found in fish mucus capable to destroy bacteria by formation of huge pores in the membranes of the target cells (Kuppulakshmi *et al.*, 2008).

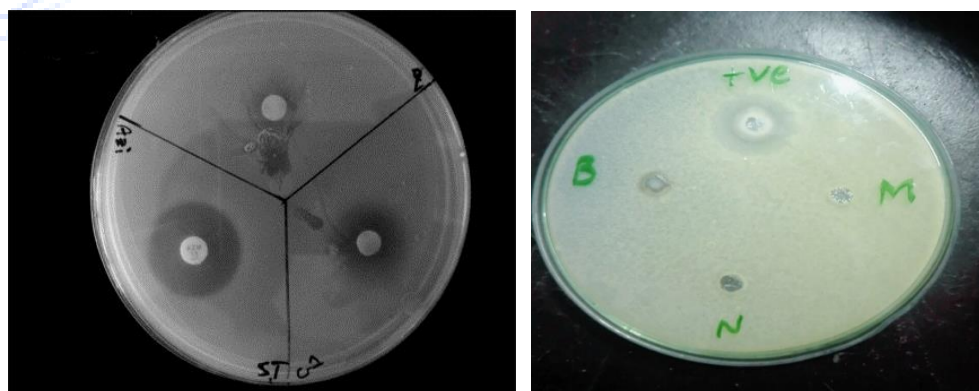
The zone of bacterial inhibition was shown in mm in table

#### Antifungal Activities:

Antifungal activities were tested against mucus extract; mucus bound Ag nanoparticles and Terbinafine. The mucus showed higher antifungal activity when compared with mucus bound Ag nanoparticles. Overall, Terbinafine exhibited highest antifungal activity. The results are given below;

**Table 4.7 Antifungal activity against different bacterial of fish (Common carp) mucus, mucus based nanoparticles and Terbinafine**

Sample	<i>A. niger</i> (mm)	<i>S. cerevisiae</i> (mm)
Mucus	14	11
Sample	10	08
Terbinafine	26	21



**Fig 4.8(a)** Antifungal activities of different samples against *A. niger* **(b)** Antifungal activities of different samples against *S. cerevisiae*.

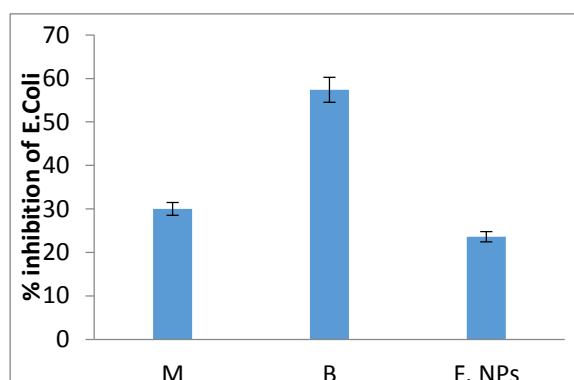
#### Minimum inhibitory concentration (MIC):

In microbiology, minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial (like an antifungal, antibiotic or bacteriostatic) drug that will inhibit the visible growth of a microorganism after overnight incubation (Wiegand *et al.*, 2008). The

MIC of two bacteria *E.Coli* and *Bacillus subtilis* was tested. The data of MIC was noted for all the samples.

**Table 4.8: MIC of mucus, mucus bound Ag nanoparticles and free nanoparticles for *E.Coli***

Sample	% inhibition
Mucus	30
Mucus based Ag nanoaprticles	57.4
Free nanoparticles	23.6



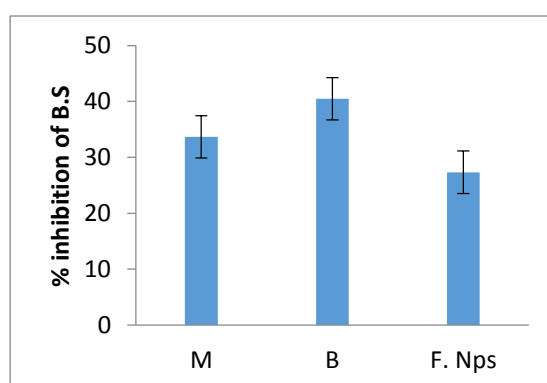
**Fig. 4.11:** Graphical representation of %inhibition of *E.Coli*

Where; M= mucus, B= Mucus based Ag nanoparticles, NPs= Nanoparticles

The data of MIC for *B.subtilis* was also noted and compiled in table.

**Table 4.9: MIC of mucus, mucus bound Ag nanoparticles and free nanoparticles for *B.subtilis***

Sample	% inhibition
Mucus	33.6666
Mucus based Ag nanoaprticles	40.4666
Free nanoparticles	27.3334



**Fig. 4.12:** Graphical representation of %inhibition of *Bacillus subtilis*

Where; M= mucus, B= mucus based Ag nanoparticles, F. NPs= free nanoparticles

The graphs represent the % inhibition of *E.coli* and *B. subtilis*. It is clear from the graph that MIC of *E.coli* is greater than that of *B.subtilis* for all the samples i.e. mucus, mucus based Ag nanoparticles and free nanoparticles. The results are supported by Rhayour *et al.* (2003) who performed MIC for

different types of essential oils and many other studies also indicated that mucus and Ag nanoparticles inhibit bacteria. He obtained the same results for these two bacteria when checked for MIC.

The results of the MIC studies by Hellio *et al.* (2002) showed that *E. coli*, *K. pneumoniae*, *S. marcescens* and *P. vulgaris* were most often inhibited by the fish extracts, all being inhibited by at least ten of the extracts studied. Conversely, *C. brusei* and *C. albicans* were inhibited by only two extracts. In the literature, some mucus extracts have been found to inhibit the growth of *E. coli* and *S. aureus* and the MICs values obtained were in the same range as ours.

Reddy *et al.* (2014) also studied the antimicrobial activity of silver oxide (Ag) nanoparticle against *Klebsiella pneumoniae*. He concluded that the standard growth curve showed that Ag Nps of 0.75 mM inhibited *K. pneumoniae* after 4 h. The interaction with outer membrane protein (OMP) and lipopolysaccharide (LPS) residues showed modulation in ~66 kDa and ~29 kDa proteins with the use of increasing concentrations of Ag Nps. The amount of nucleic acid and protein released from the cells increased with the Ag Nps concentration used. Importantly, the OD of the Ag Nps-treated cells decreased within 30 min of incubation in the presence of SDS. Ag Nps-treated *K. pneumoniae* were five-fold less infectious in the HEp-2 cell line at doses between 0.50 and 0.75 mM.

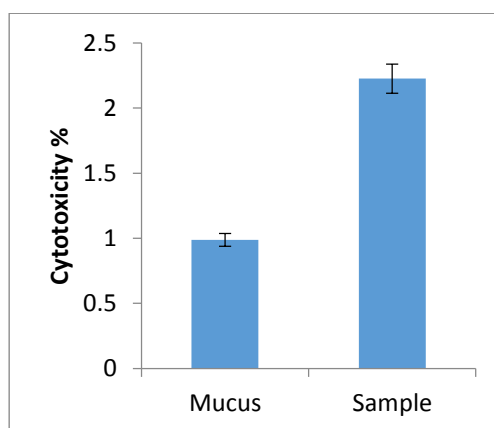
#### (4) Cytotoxic Activity

##### Hemolytic activity:

This essay is used to check the hemolysis of different samples. EDTA was used to save the bleeding from clotting. The percentage hemolysis of crude mucus extract are shown in below graph and in table number 4.3. The more hemolytic activity was measured in crude mucus extract of common carp that is 40%. As a positive control Triton-X was used and its percentage hemolysis was 90%.

**Table 4.10: Hemolytic assay of mucus and mucus based Ag nanoparticles. Given data is the average of three replicates±S.D**

Sample	Hemolytic activity
Mucus	0.98±0.31
Sample	2.22±0.49



**Fig 4.13** Cytotoxicity of fish mucus extract against human RBC

The graph represents that the hemolytic activity of mucus and mucus based Ag nanoparticles. It is obvious that the hemolytic activity of mucus is less than that of the mucus based Ag nanoparticles.

Zhong *et al.* (2013) studied the hemolytic assay of snail mucus on human red blood cells. He concluded that Some AMPs were found to exert hemolytic activities. Human red blood cells were used

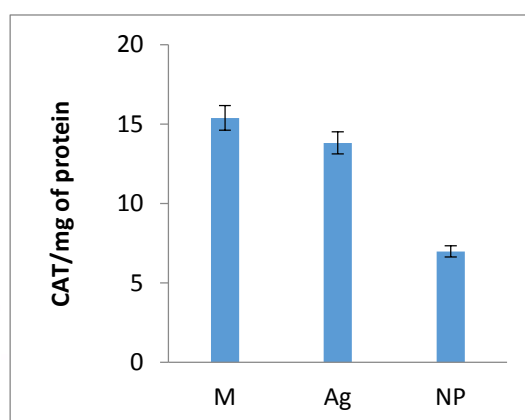
to evaluate the peptide's hemolytic capability. The result showed little hemolytic activity was exerted by mytimacin-AF. At the concentration of 5, 10, 20, 40, 80, 160, and 320  $\mu\text{g/ml}$ , respectively, it induced 1.1, 1.3, 1.7, 2.4, 2.3, 3.2, and 3.9% human red blood cell hemolysis, respectively.

### (5) Biochemical analysis

Biochemical analysis of fish mucus was done by Catalase (CAT), superoxide dismutase (SOD) and peroxidase (POD) assays along with protein estimation.

#### (a) Catalase (CAT)

In this case  $\text{H}_2\text{O}_2$  was used as a substrate and the decomposition of  $\text{H}_2\text{O}_2$  by the catalase enzyme was observed using UV-vis spectrophotometer. The absorbance measured at 240 nm.



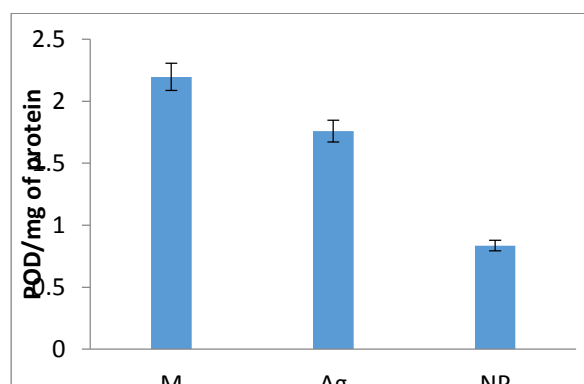
**Fig 4.14** Graphical representation of comparative analysis of catalase activity of mucus, free Ag nanoparticles and sample.

Where; M= Mucus, Ag= Mucus based Ag nanoparticles, NPs= Nanoparticles

The catalase activity was measured in mucus, mucus based Ag nanoparticles and free nanoparticles. The results indicated that the catalase activity is highest in mucus. Mucus based Ag nanoparticles also exhibit noticeable catalase activity while free nanoparticles showed less activity when compared with other groups as indicated by graph above;

#### (b) Peroxidase activity (POD):

The POD activity was assayed using guaiacol as a hydrogen donor by measuring the change at 470 nm.



**Fig 4.15** Graphical representation of peroxidase activity

Where; M= Mucus, Ag= Mucus based Ag nanoparticles, NPs= Nanoparticles

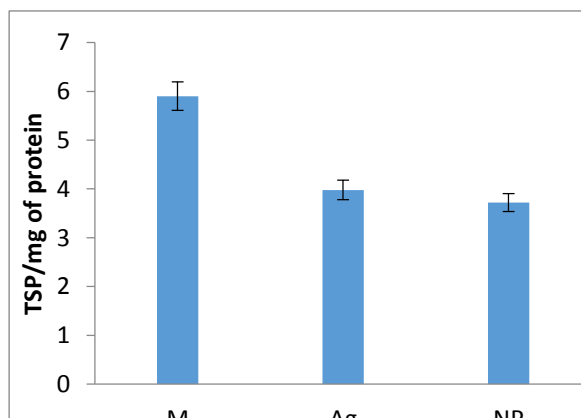
The peroxidase activity was measured in mucus, mucus based Ag nanoparticles and free nanoparticles. The results indicated that the peroxidase activity of mucus is highest. Mucus based Ag nanoparticles also displayed peroxidase activity while free nanoparticles showed less activity when compared with other groups as indicated by graph above;

**Table 4.11 TSP, CAT, POD and SOD values of mucus, mucus based Ag nanoparticles and free nanoparticles. Given data is the value of triplicates  $\pm$  SD.**

Sample	TSP	CAT	POD	SOD
Mucus	5.90 $\pm$ 0.13	15.28 $\pm$ 0.70	2.19 $\pm$ 0.18	10.05 $\pm$ 0.04
Mucus based Ag nanoparticles	3.97 $\pm$ 0.17	14.92 $\pm$ 0.36	1.75 $\pm$ 0.82	10.39 $\pm$ 0.11
Ag nanoparticles	3.72 $\pm$ 0.01	5.24 $\pm$ 0.58	0.83 $\pm$ 0.99	77.28 $\pm$ 0.64

**(c) Protein estimation:**

In protein estimation assay samples were diluted to obtain protein. Bovin serum albumin used as standard. The standard was prepared containing a range of 200 to 2000 micrograms protein (Bovine serum albumin 2 mg/ml in 1000  $\mu$ l volumes for setting up the standards). The absorbance (OD) was measured at 595 nm with the help of spectrophotometer.



**Fig 4.16** Graphical representation of total soluble protein.

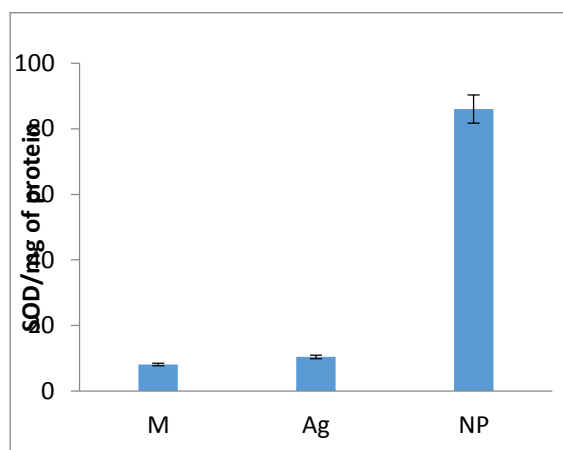
Where; M= Mucus, Ag= Mucus based Ag nanoparticles, NPs= Nanoparticles

The total soluble protein was measured in mucus, mucus based Ag nanoparticles and free nanoparticles. The results indicated that the amount of total soluble proteins is highest in mucus. Mucus based Ag nanoparticles also have considerable amount of proteins while free nanoparticles have less proteins as indicated by graph above

**(d) Superoxide Dismutase (SOD):**

The reagents used in SOD assay included phosphate buffer (pH 7.5), riboflavin, nitro blue tetrazolium, Triton-X and methionine. After exposure of 15 min in UV light added riboflavin at the end. The absorbance was measured at 560 nm.





**Fig 4.17** Graphical representation of superoxide dismutase activity

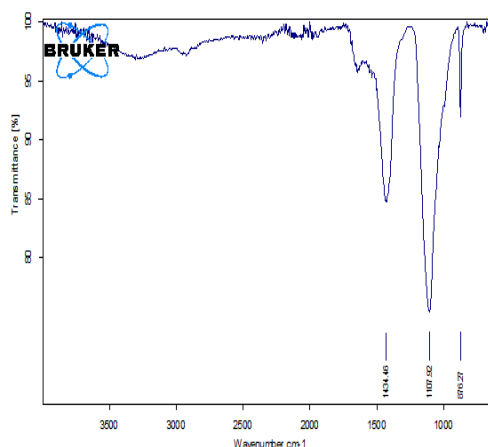
Where; M= Mucus, Ag= Mucus based Ag nanoparticles, NPs= Nanoparticles

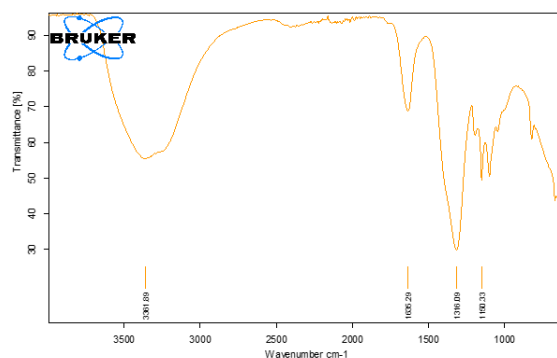
The superoxide dismutase activity was measured in mucus, mucus based Ag nanoparticles and free nanoparticles. The results indicated that the superoxide dismutase activity of mucus is highest. Mucus based Ag nanoparticles also displayed superoxide dismutase activity while free nanoparticles showed less activity when compared with other groups as indicated by graph below;

#### (6) Characterization:

##### (a) Fourier infrared spectroscopy:

(a) In the FT-IR spectrum different peaks were observed of mucus, binding material and the nanoparticles. Each spectrum of the sample gave different peaks. The peaks observed for dry mucus contain different functional groups at various wavelengths. FTIR spectrum shows functional groups alkane, alkyl amine and alkyl halides at wavelengths of 1434.16, 1107.92, 876.27 and 618.44 respectively. (b) The FTIR spectrum of mucus based Ag nanoparticles shows four peaks at different wavelengths. The functional groups are secondary amines (N-H), alkanes, alkyl ketones and alkyl amine at wavelength of 3361.89, 1635.29, 1316.09 and 1150.33 respectively.

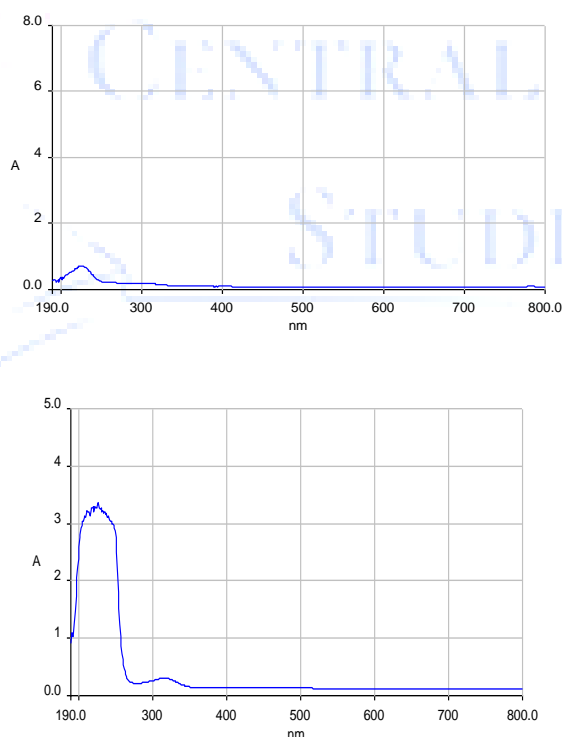




**Fig 4.18(a)** Graphical representation of FTIR spectra of mucus **(b)** Graphical representation of FTIR spectra of mucus based Ag nanoparticles

#### **(b) UV spectra**

Normal range of UV-vis spectra used is ranged from 200 nm to 1100 nm through which peaks of different functional groups are found. In this spectra the maximum peak observed at 250 nm and the lowest peak observed at 1100 nm (0.5nm). The observed spectrum peak is highest at between 200 and 300 nm but after 300 nm the peaks begin to decline. The maximum absorbance is at 250 nm.



**4.20(a)** Graphical representation of UV spectra. Crude mucus of carp **(b)** Graphical representation of UV spectra, mucus based nanoparticles

The peak of crude mucus extract was between 190-300 nm and same is true for mucus based Ag nanoparticles. The difference lies in the length of the peak. Crude mucus extract showed a peak at the length of 1-2 while mucus based Ag nanoparticles had a peak at the length between 3-4.

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